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Increased Frequency of HLA DR13 in Hepatitis C Virus Carriers With Persistently Normal ALT Levels

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The aim of this study was to clarify the relationship between human leukocyte antigen DR allele distribution and the degree of liver cell injury of hepatitis C virus (HCV) carriers in Japan. The subjects, 68 HCV carriers, were divided into two groups according to the laboratory data and liver histology. Those in the asymptomatic carrier group (n = 19) had normal ALT levels persistently for 8–153 months (mean 25.7 months) and were diagnosed histologically as normal liver, nonspecific reactive hepatitis or chronic persistent hepatitis. Those in the chronic active hepatitis group (n = 49) had elevated ALT levels and were diagnosed histologically with chronic active hepatitis. The human leukocyte antigen DR alleles of all subjects were defined using the polymerase chain reaction restriction fragment length polymorphism method. The expression of human leukocyte antigen class I antigen and intercellular adhesion molecule 1 on the hepatocyte membrane were also examined in 14 patients from each group using an indirect immunohistochemical method. The frequency of DR13 (42.1%) in the asymptomatic carrier group was significantly higher ($Pc < 0.003$) than that of the chronic active hepatitis group (4.1%). There were no significant differences for the other DR alleles. The frequencies of expression of human leukocyte antigen class I antigen and intercellular adhesion molecule 1 on the hepatocyte membrane of the asymptomatic carrier group were significantly less than those of the chronic hepatitis group (64% vs. 100% $P < 0.05$, 29% vs. 71% $P < 0.05$, respectively), although there was no significant difference in the serum HCV-RNA titer between the two groups ($10^{6.4\pm1.1}$ vs. $10^{6.5\pm0.7}$ copies/mL). These results demonstrate that the cellular immune response of the asymptomatic carrier group is less activated than the response of the chronic active hepatitis group and that HLA DR13 may be closely associated with this low activity of hepatitis among HCV carriers.

KEY WORDS: asymptomatic carrier, HLA DNA typing, HLA class I antigen, HLA class II DR antigen, cellular immunity, ICAM-1

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of post-transfusion hepatitis [Choo et al., 1989; Kuo et al., 1989] and is one of the etiologic agents of chronic hepatitis, which is associated with the development of cirrhosis and hepatocellular carcinoma [Kiyosawa et al., 1990]. However, among HCV carriers, there are some individuals who display no symptoms or signs of liver damage [Alberti et al., 1992; Brillanti et al., 1993]. Some conditions of HCV, such as the amount of HCV-RNA, HCV genotype, and the degree of mutation, have been suggested to be important factors influencing the clinical course of infection [Okamoto et al., 1992; Hagiwara et al., 1993a; Kato et al., 1993; Mita et al., 1994a] and the efficacy of interferon therapy [Yoshioka et al., 1992; Hagiwara et al., 1993b; Kanazawa et al., 1994; Mita et al., 1994b]. Although it is becoming clearer that T-cell-mediated immune reactions [Koziel et al., 1992; Botarelli et al., 1993; Ferrari et al., 1994] and Fas antigen expression [Hiramatsu et al., 1994] play crucial roles in the mechanism of liver injury in type C chronic hepatitis, the factors that regulate host immunity are still unclear. Human leukocyte antigen (HLA) molecules play an important role in the immune reaction

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TABLE I. Characteristics and HLA DR Antigens in HCV Carriers With Persistently Normal ALT Levels

Patient no.[a]	Age/sex (yr)	ALT (IU/L)	Time since transfusion (yr)	Follow-up (Mo)	HCV-RNA Titer[b]	Genotype	Histological diagnosis[c]	HAI SCORE I	II	III	IV	HLA-DR locus
1	66 M	11.2	20[d]	8	3.5	1b	NL	0	0	0	0	12/13
2	54 F	17.8	37	20	8	1b	NL	0	0	0	0	4/13
3	57 F	22.1	—	15	6	1b	NL	0	0	0	0	8/10
4	52 M	17.5	30[d]	15	7	2b	NL	0	0	0	0	2/4
5	62 M	15.0	—	19	7	2a	NL	0	0	0	0	4/8
6	56 M	15.0	38	14	7	1b	NSRH	0	0	1	0	13/14
7	58 F	21.0	41	33	8	1b	NSRH	0	0	1	0	11/13
8	48 F	22.1	—	19	7	1b	NSRH	0	0	1	0	2/13
9	57 M	25.1	3	15	5.5	1b	NSRH	0	1	0	0	4/11
10	26 F	28.0	13	17	7	1b	NSRH	0	0	1	0	1/4
11	46 F	30.2	20	9	5.5	1b	NSRH	0	0	1	0	1/1
12	67 F	12.9	—	153	7	2a	NSRH	0	0	1	0	4/13
13	44 F	15.6	—	25	7	2b	NSRH	0	0	1	0	2/4
14	46 F	18.0	20	10	7	1b	CPH	0	1	1	0	8/13
15	72 M	15.9	15	28	6	1b	CPH	0	1	1	0	4/13
16	66 M	10.6	—	17	7	1b	CPH	0	1	1	0	4/12
17	65 F	17.3	—	23	7	1b	CPH	0	1	1	1	9/12
18	60 F	16.3	—	23	6	1b	CPH	0	0	3	0	4/11
19	65 F	15.8	—	25	6	2a	CPH	0	0	3	1	9/14

[a]All but nos. 2, 5, 16, 17, 19 were examined immunohistochemically.
[b]HCV-RNA titer:$\log_{10}$ copies/mL.
[c]NL = normal liver, NSRH = nonspecific reactive hepatitis, CPH = chronic persistent hepatitis.
[d]Has tattoos on back.

and recently have been suggested to be strongly associated with the clinical profile or course of several diseases, especially autoimmune diseases such as Graves disease [Schifferdecker et al., 1991], ulcerative colitis [Toyoda et al., 1993], rheumatoid arthritis [Grennan et al., 1986], and autoimmune hepatitis [Donaldson et al., 1991; Czaja et al., 1993; Marcos et al., 1994].

The polymerase chain reaction (PCR) procedure [Saiki et al., 1985, 1988], which enables specific amplification of the targeted genetic region by using Taq DNA polymerase, has made possible more accurate typing of HLA as compared to serotyping. The aim of this study was to clarify the relationship between HLA DR allele distribution examined by the PCR restriction fragment length polymorphism (PCR-RFLP) method and clinical and histological features. In the portal area, lymphocyte infiltration is observed not only in patients with chronic active hepatitis, but also in carriers with persistently normal ALT levels. However, it is not known whether there are differences between them in the cellular immune response on hepatocytes. Therefore, in order to evaluate the activity of cellular immunity, the expression of HLA class I antigen and intercellular adhesion molecule 1 (ICAM-1) were investigated in liver tissue from HCV carriers using indirect immunohistochemical staining, because the hepatocyte expression of HLA class I antigen and ICAM-1 was suggested to play an important role in the cellular immune response of chronic viral hepatitis [Chu and Liaw, 1993; Mosnier et al., 1994].

MATERIALS AND METHODS

Patients

A total of 68 patients (36 males and 32 females) were studied, all of whom were positive for anti-HCV by second-generation ELISA and serum HCV-RNA by RT-PCR. None of the patients had any evidence of chronic hepatitis B (negative for HBs antigen and antibody to HBc antigen and autoimmunity (negative for antinuclear antibody). They were divided into two groups, according to the laboratory data and liver histology. Those in the asymptomatic carrier group (7 males and 12 females, ages 26–72 yr, mean 56.2 ± 10.9 yr) had normal ALT levels persistently for 8–153 months (mean 25.7 months) and were diagnosed histologically with normal liver, nonspecific reactive hepatitis or chronic persistent hepatitis. Eight patients in the asymptomatic carrier group had histories of blood transfusion (3–41 yr ago) and two patients had tattooes on their backs. Details of the clinical data are shown in Table I. Those in the chronic active hepatitis group (25 males and 24 females, ages 29–66 yr, mean 53.4 ± 8.7 yr) had elevated ALT levels and were diagnosed histologically with chronic active hapatitis. Liver biopsy was carried out for diagnostic purposes, and informed consent was obtained from each patient. The specimens were also evaluated according to the histological activity index (HAI) scoring system by Knodell et al. [1981]. Liver tissue samples from 28 randomly selected patients (14 patients of each group) were examined immunohistochemically. Half of the liver specimens were used for routine histological examination and the other half for immunohistochemical staining.

HLA DR DNA Typing

Genomic DNAs from peripheral blood cells were isolated by proteinase K-SDS digestion followed by phenol-chloroform extraction according to previous reports [Inoko et al., 1986; Kaneshige et al., 1992]. HLA-DR typing by PCR-RFLP was carried out as described previously [Ota et al., 1992]. Genomic DNA (200–300 ng) was amplified by the PCR procedure with 2.5 units of

Taq DNA polymerase (Perkin Elmer Cetus, Emeryville, CA). The reaction mixture (genomic DNA, PCR buffer; 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.4, 0.01% gelatin, 0.02% N(Nonidet)P-40; 200 μ M each of dATP, dCTP, TTP, and dGTP; 1 μ M of each of the primers) and distilled water for a total volume 100 μl in a 1.5 ml Eppendorf tube was covered with 50 μl of mineral oil to prevent evaporation and then subjected to 30 cycles of 1 min at 94°C, 1 min at 62°C, and 2 min at 72°C by automated PCR thermal sequencer. For DR1 and DR9 amplification, annealing was performed at 55°C. The second exon of the DRB1 gene was amplified using one of seven group-specific 5′-primers (5′TTCCTGTGGCAGCCTAAGAGG3′ for DR2, 5′GTTTCTTGGAGCAGGTTA AAC3′ for DR4, 5′GAAGCAGGATAAGTTTGAGTG3′ for DR9, 5′GGTTGCTGGAAA GATGCATCT3′ for DR1, 5′AGTTCCTGGAAAGACTCTTCT3′ for DR7, 5′GGTTGCT GGAAAGACGCGTCC3′ for DR10 and 5′ACGTTTCTTGGAGTACTCTACG3′ for DR3, 5, 6, 8), along with the common 3′-primer (3′CCGCTGCACTGTGAAGCTCT). After amplification, aliquots (7 μl) of the reaction mixture were digested with restriction endonucleases (AvaII and PstI for DR1-DRB1; FokI, Cfr13I and HphI for DR2-DRB1; SacII, AvaII, HinfI, HaeII, HphI, and MnlI for DR4-DRB1; AvaII, FokI, KpnI, HaeII, Cfr13I, SfaNI, SacII, BsaJI, ApaI, HphI, and RsaI for DR3, 5, 6, and 8-DRB1) for 1–3 h after addition of an appropriate incubation buffer at suitable temperature. Complete digestion of restriction enzymes was confirmed by including positive control DNAs with the HLA alleles, which have cleavage sites for the respective enzymes in the PCR-amplified regions. Samples of the restriction enzyme-cleaved amplified DNAs were usually subjected to electrophoresis in 12% polyacrylamide gels in a horizontal minigel apparatus (Mupid, Cosmo Bio Co., Tokyo, Japan). Whether or not the amplified fragments were cleaved was detected by staining with ethidium bromide.

Immunohistochemical Procedures

Fourteen liver samples from each group were obtained by percutaneous needle biopsy using a 15-gauge Trucut needle. Fragments of specimens were snap frozen in liquid nitrogen cooled with isopentane and stored at −80°C until use. The cryostat sections (6 μm) were dried overnight at room temperature and fixed in acetone at 4°C for 3 min, followed by extensive washing with phosphate-buffered saline (pH 7.2) before staining. Briefly, the sections were incubated with 1:100 diluted anti-HLA-class I antibody (DAKO A/S Distributors, Glostrup, Denmark), and 1:50 diluted anti-ICAM 1 (CD54) antibody (DAKO A/S Distributors). Next, they were incubated for 20 min with biotinylated 1:20 rabbit antimouse immunoglobulin (Ig G), then avidin-biotin peroxidase complex was applied (Vectastain ABC kits Vector Laboratories, Burlingame, CA), followed by diaminobezidine-H_2O_2 substrate and counterstaining with hematoxylin. The immunostaining was also done without the primary antibodies for negative control. Samples were preincubated with 0.3% hydrogen peroxide in methanol. The expression of ICAM-1 and HLA class I antigen on the hepatocyte membrane was evaluated as negative or positive: negative, staining was present on the sinusoidal lining cells but absent from the hepatocyte membrane; positive, staining was more or less positive on the hepatocyte membrane.

Competitive RT-PCR Assay and Typing HCV Genotype

Competitive RT-PCR was carried out as described previously [Hagiwara et al., 1993]. In brief, extracted HCV-RNA was reverse transcribed with known amounts of mutant HCV-RNA with a novel restriction endonuclease (EcoRI) site in the sequence of 5′-UTR. The cDNAs derived from wild and mutant HCV-RNA were co-amplified with 10 pmol each of external primers for 40 cycles of PCR (94°C for 30 sec, 60°C for 1 min, and 72°C for 1 min) and were treated with EcoRI (Toyobo Co., Osaka, Japan). The amounts of HCV-RNA were determined by comparing the signal intensities of PCR products derived from the targeted RNA with those from mutant RNA after staining with ethidium bromide and UV light observation. HCV genotypes were determined by a method described previously [Mita et al., 1994b]. HCV was classified into four genotypes (1a, 1b, 2a, and 2b) based on variation in the nucleotide sequence within restricted regions in the putative HCV core gene [Simmonds et al., 1994]. Briefly, a core region was amplified by PCR using a universal primer pair, and then selective amplification of the first PCR products was performed using a nested pair of genotype-specific antisense primer and a universal sense primer. The genotype-specific primers were chosen to amplify a different size sequence for each genotype. The products of the second-stage PCR were subjected to electrophoresis in agarose, and HCV genotypes were identified by the length of the amplified sequence.

Statistical Analysis

Values are expressed as mean ± S.D. The means were compared by the Mann-Whitney U test and the χ^2 or Fisher's exact test where appropriate. A level of $P < 0.05$ was accepted as being statistically significant. The corrected P value (Pc) was calculated by multiplying the P value by the number of comparisons made. A level of $Pc < 0.05$ was accepted as being statistically significant.

RESULTS

HLA DR DNA Typing and HCV Genotype

The HLA DR frequency was investigated in the asymptomatic carrier group and the chronic active hepatitis group. There was no significant difference in age between the two groups. The frequency of DR13 of the asymptomatic carrier group was significantly higher than that of the chronic active hepatitis group (42.1% vs. 4.1% $Pc < 0.003$), whereas there were no significant differences with the other DR alleles. HCV genotype 1b was observed in 14 of 19 cases (74%) in the asymptomatic carrier group (Table II). If only those with genotype

TABLE II. Characteristics and Frequencies of HLA DR Antigens in Asymptomatic Carrier and Chronic Active Hepatitis Groups

Clinical features and HLA DR typing	Asymptomatic carrier[a] (n = 19)	Chronic active hepatitis (n = 49)
Male/female	7/12	25/24
Age (yr)*	56.2 ± 10.9**	53.4 ± 8.7
ALT (IU/L)*	18.3 ± 5.3***	121.1 ± 81.4
DR antigens (%)		
DR1	10.1	26.5
DR2	15.8	36.7
DR4	47.3	63.3
DR11	15.8	2.0
DR12	15.8	12.1
DR13	42.1****	4.1
DR14	10.1	12.2
DR8	15.8	18.4
DR9	10.1	14.3
DR10	5.3	2.0

[a]Carrier with persistently normal ALT level.
*Data expressed as mean ± S.D.
**No significant difference vs. patients with chronic hepatitis (Mann-Whitney U test).
***$P < 0.001$ vs. patients with chronic hepatitis (Mann-Whitney U test).
****$Pc < 0.003$ vs. patients with chronic hepatitis (Fisher's exact test).

1b were considered, interestingly, the frequency of patients with DR13 increased (7 of 14 cases, 50%), whereas the two patients with HCV genotype 2b both had DR2/4 and three patients with HCV genotype 2a had DR4/8, DR4/13 or DR9/14 (Table I).

Immunohistochemistry

With 28 patients (14 patients from each group) selected at random, we examined immunohistochemically (Fig. 1) the expression of HLA class I antigen and ICAM-1 on the hepatocyte membrane (Table III). HLA class I antigen and ICAM-1 were expressed on varying proportions of hepatocyte membrane throughout the portal areas and lobular parenchyma, the sinusoidal lining cells, and the mononuclear inflammatory cells. In all liver samples of the chronic active hepatitis group, HLA class I antigens were expressed mainly on the hepatocyte membrane, whereas the expression of HLA class I antigen on the hepatocyte membrane was observed in 64% of the asymptomatic carrier group. ICAM-1 was also expressed on the hepatocyte membrane, and the pattern was similar to that of the HLA class I antigen. The frequencies of expression of ICAM-1 on the hepatocyte membrane in the asymptomatic carrier and chronic active hepatitis groups were 29% and 71%, respectively (Table III). On the whole, the frequencies of expression of HLA class I antigen and ICAM-1 on hepatocyte membrane of the asymptomatic carrier group were significantly less than that of the chronic active hepatitis group. In contrast, there were no significant differences in patient age and serum HCV-RNA titer ($10^{6.4\pm1.1}$ vs. $10^{6.5\pm0.7}$ copies/mL) between the two groups (Table III).

DISCUSSION

Previous studies have shown that the amount of HCV-RNA titers, genotype, and the degree of mutation of HCV-RNA influence on the outcome of the natural course of infection [Okamoto et al., 1992; Hagiwara et al., 1993a; Kato et al., 1993; Mita et al., 1994a] or the efficacy of interferon therapy [Yoshioka et al., 1992; Hagiwara et al., 1993b; Kanazawa et al., 1994; Mita et al., 1994b] in HCV carriers. However, the factors that determine the outcome of chronic HCV infection still remain unclear. The interesting finding of this study is that patients with low activity of both biochemical data and liver histology more frequently had DR13 than those with active hepatitis. Furthermore, the expression of intercellular adhesion molecules that play an important role in stabilizing cell-cell recognition [Springer, 1990] was significantly less than that of the chronic active hepatitis group. Taken together, these facts indicate that in type C chronic hepatitis, regulation of the T-cell-mediated immune response plays an important role in liver injury and that a low activity for the immune response may be closely associated with DR13.

Differences in the immune response among individuals are known to be derived from allelic variations in HLA antigens, especially DR antigen [Ceppellini et al., 1989; Blackman et al., 1990], which act as antigen-presenting molecules on antigen-presenting cells at the time of induction of the immune reaction when foreign antigens or self-antigens are presented to T lymphocytes. In the asymptomatic carrier group, 42.1% of the individuals had DR13, which is observed at 6.2% (allele frequency) in a Japanese population (n = 916) [Hashimoto et al., 1994], and if only those with HCV genotype 1b were considered, the frequency of DR13 increased to 50%, whereas the two patients with genotype 2b had DR2/4 and the three patients with genotype 2a had DR 4/8, 4/13, or 9/14. These results indicated that a combination of DR13 and some HCV antigen, especially in the case of genotype 1b, makes it difficult to induce the T-cell-mediated immune response. At present, the precise mechanism of this condition remains unclear. It can be explained by two assumptions. One is that DR13 molecules do not bind HCV peptides easily nor do they easily form a complex. The other is that they can bind and form the complexes, but the complexes have little or no ability to activate the helper T cell 1 (Th 1), which induces the cell-mediated immune response [Mosmamm et al., 1986], whereas they are able to activate the helper T cell 2 (Th 2), which induces the humoral immune response [Swain et al., 1988]. In many HCV asymptomatic carriers, the humoral immune response seems to be activated, because most were positive for HCV antibody, and this evidence supports the latter assumption.

The frequencies of DR1, DR2, and DR4 in the chronic active hepatitis group tended to increase compared with those in the asymptomatic carriers group (Table II), although there were no significant differences be-

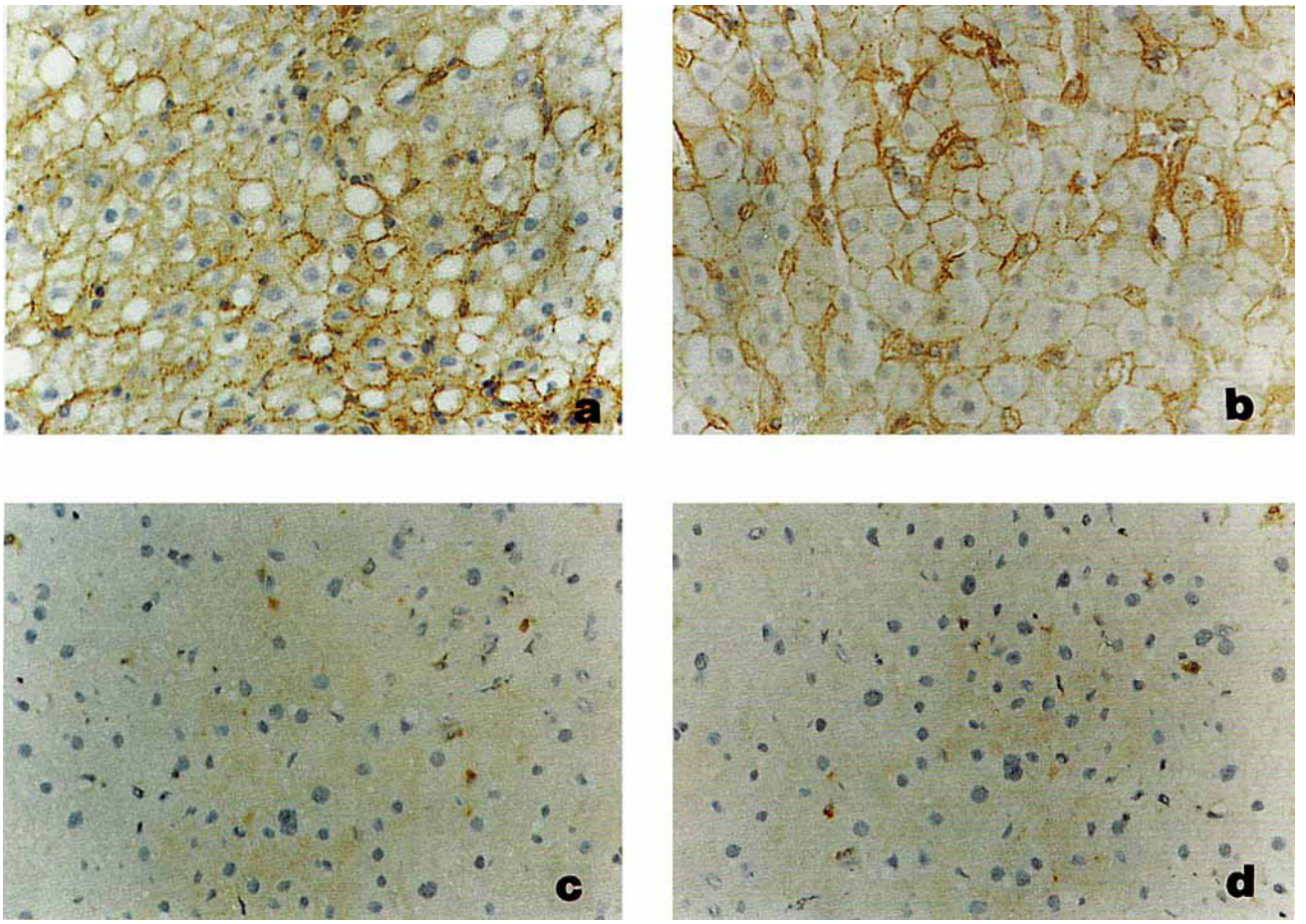

Fig. 1. Immunoperoxidase staining of liver biopsy sections with anti-HLA class I and anti-ICAM-1 of representative cases. HLA class I antigen was expressed strongly on the hepatocyte membrane and moderately on the sinusoidal lining cells (a patient with chronic active hepatitis) **(a)** (×400). A similar expression pattern was observed in the section with anti-ICAM-1 (a patient with chronic active hepatitis) **(b)** (×400). In contrast, HLA class I antigen **(c)** and ICAM-1 **(d)** were expressed slightly or not at all on sinusoidal lining cells and not on the hepatocyte membrane (a patient with persistently normal ALT levels) (×400).

tween the two groups. In this study, the number of patients might be too small to establish whether they were significantly associated with the progression of liver disease in HCV carriers. As DR4 has been reported to be one of the risk factors in autoimmune hepatitis [Donaldson et al., 1991; Marcos et al., 1994], there is a need to examine this using a larger number of patients.

Several reports indicated that the amount of serum HCV-RNA was significantly lower in patients with chronic persistent hepatitis than in those with chronic active hepatitis or liver cirrhosis, and it tended to increase according to the progression of histopathological changes of the liver [Hagiwara et al., 1993a; Kato et al., 1993]. This tendency was also observed in HCV carriers were persistently normal ALT levels [Naito et al., 1994], suggesting that the amount of HCV-RNA became larger exponentially as the term of infection became longer [Kato et al., 1993]. In the present study, no significant difference was found in serum HCV-RNA titer between the asymptomatic carrier and chronic active hepatitis groups. We searched for HCV asymptomatic carriers among blood donors using the HCV antibody screening test and carefully selected carriers with persistently normal ALT levels by means of long-term follow-up. Consequently, the age of our patients with persistently normal ALT levels was similar to that of our patients with chronic active hepatitis, and we assumed that the asymptomatic carrier group had nearly the same term of infection as the chronic active hepatitis group. This might be one reason that there was no significant difference in the serum HCV-RNA titer between the two groups.

In conclusion, a strong association of HLA DR13 with low activity of hepatitis was observed in HCV carriers, and the expression of HLA class I antigen and ICAM-1 on the hepatocyte membrane of patients with persistently normal ALT levels were significantly less than that of patients with chronic active hepatitis, although there was no significant difference in the serum HCV-RNA titer between them. These results indicate that the T-cell-mediated immune response plays an impor-

TABLE III. Characteristics and Expression of HLA Class I and ICAM-1 on Hepatocyte Membrane in HCV Carriers

Clinical feactures and expression of hepatocyte membrane	Asymptomatic carrier[a] (n = 14)	Chronic active hepatitis (n = 14)
Male/female	5/9	9/5
Age (yr)*	53.9 ± 11.7**	49.9 ± 11.2
HCV-RNA titer* (log_{10}copies/mL)	6.4 ± 1.1**	6.5 ± 0.7
ALT(IU/L)*	19.4 ± 5.6****	109.1 ± 58.6
HCV genotype (n)	1b (11) 2a (1) 2b (2)	1b (8) 2a (4) 2b (2)
DR13 positive patients (%)	7/14*** (50)	1/14 (7)
HLA class I positive (%)	9/14*** (64)	14/14 (100)
ICAM-1 positive (%)	4/14*** (29)	10/14 (71)

[a]Carrier with persistently normal ALT level.
*Data expressed as mean ± S.D.
**No significant difference vs. patients with chronic hepatitis (Mann-Whitney U test).
***$P < 0.05$ vs. patients with chronic hepatitis (Fisher's exact test).
****$P < 0.001$ vs. patients with chronic hepatitis (Mann-Whitney U test).

tant role in liver injury, and DR13 may be a maker for low hepatitis activity among HCV carriers. Further investigations and careful follow-up are needed to confirm this. According to a recent report, among the HLA antigens, the DP molecule plays a primary and decisive role in the immunogenetic pathogenesis of early-onset Graves disease [Omura et al., 1994]. Further studies are needed on the typing of HLA DP, DQ antigens and haplotypes among HCV carriers.

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